

DETERMINING MAINTENANCE ENERGY REQUIREMENTS OF RUMEN PROTOZOA  
TO IMPROVE RUMINANT LIVESTOCK FEED EFFICIENCY

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## INTRODUCTION

Ruminants (cattle, sheep, goats) are able to digest and convert fibrous feedstuffs into meat, milk, and fiber due to the presence of microbes that inhabit their rumen (forestomach). These microbes, which include bacteria, protozoa, fungi, and archaea, anaerobically degrade feed and produce ATP and fermentative end-products, primarily volatile fatty acids. With this ATP, the microbes synthesize cellular protein that comprises up to 90% of the amino acids that reach the small intestine of the ruminant host (Russell and Rychlik, 2001). Better understanding of the growth efficiency of rumen microbes would improve prediction of amino acids available for digestion, increase the confidence in formulating diets, and eliminate or reduce the excess protein that is currently fed as a safety margin to most ruminants in order to make sure nutrient requirements are met.

Because excess protein is fed as a safety factor, high amounts of RDP are converted to ammonia, which diffuses into the bloodstream. As a result, 70 to 90% of the nitrogen (N) in feed is excreted in manure (Rotz, 2004). Globally, this equates to  $70 \cdot 10^8$  kg N/yr, including  $7.9 \cdot 10^8$  kg N/yr for U.S. dairy cattle operations alone (St-Pierre and Thraen, 1999) and justifies the need to better understand growth efficiency of rumen microbes in order to reduce N excretion into the environment. Reducing the amount of protein in livestock feed would also reduce feed costs for producers due to the high cost of feed protein. High-quality protein sources such as blood meal can cost over twice the price of low-protein sources, such as corn or other “energy” sources that could grow cheaper microbial protein in the rumen (Anonymous, 2011).

Microbes do not grow with perfect efficiency because they divert some energy towards maintenance and waste some energy via heat production; when heat production increases to intentionally dissipate ATP, this process is termed energy spilling (Tempest and Neijssel, 1984).

Maintenance functions include resynthesis of protein after turnover and establishment of ion gradients (Russell, 2007). Cells require a constant amount of energy for maintenance, and this is equal to energy use when the cells are not growing (Pirt, 1965). Therefore, cells growing at a slow rate will divert a large proportion of energy towards maintenance. In rumen bacteria, maintenance accounts for 25 to 40% of total energy use. Growth efficiency thus ranges from 13 to 19 g dry cells/mol ATP produced, which is only 60 to 80% of the maximum obtainable (Fig. 1). Clearly, maintenance requirements can place a large drain on growth efficiency.

Maintenance energy has been studied in rumen bacteria but not protozoa, likely because of difficulties in culturing protozoa and the inability to culture them independent of bacteria that are required sources of nutrients (Dehority, 2003). Rumen protozoa make up 10 to 50% of the microbial biomass in the rumen (Coleman, 1979) but are largely ignored in ruminant protein models (NRC, 2001) primarily because of lack of quantitative knowledge of their role in protein supply to the small intestine. In contrast to dogmatic views of protozoal retention and lysis in the rumen, their growth is rapidly modified to meet changes in substrate supply in relation to their population density (Sylvester et al., 2009).

The initial aim of this study was to quantify maintenance energy of a predominant protozoal species (*Entodinium caudatum*) by measuring heat production across different growth rates and extrapolating energy usage at a growth rate of 0. Protozoa cannot live without consuming and digesting bacteria for growth factors, so the literature is very sparse for physiological measurements of rumen protozoa. We estimated a maintenance requirement of 30-40 mW/ g microbial protein based on bacterial requirements from Isaacson et al. (1975) and endogenous fermentation of hexose by protozoa (Dijkstra, 1994). The value of energy use of rumen bacteria extrapolated to growth rate = 0 was set equal to 28.0 mW/g protein, which was

calculated from Isaacson (1975) [ $2.6 \cdot 10^{-4}$  mol glucose cell  $\text{DM}^{-1} \text{h}^{-1}$  reported by Isaacson (1975) x 196.7 kJ/mol hexose (Hackmann et al., 2013) x 1 g DM/0.507 g protein (Hackmann et al., 2013) x 1 h/3600 s x  $10^6$  mJ/kJ]. Expected energy use by protozoa extrapolated to growth rate = 0 was set equal to 38.7 mW/g protein, calculated from Dijkstra (1994) [8.5 mmol hexose g protozoa  $\text{DM}^{-1} \text{d}^{-1}$  reported by Dijkstra (1994) x 1 g DM/0.507 g protein (Hackmann et al., 2013) x 199.4 kJ/mol hexose (Hackmann et al., 2013) x 1 d/86,400 s x  $10^{-3}$  mol/ mmol].

Based on early results in which heat production was so close to the sensitivity limit of our instrumentation, the original protocol was adjusted from using a culture of *E. caudatum* to quantify maintenance energy to using mixed protozoal cultures from the rumen to quantify endogenous metabolism by measuring heat production of starved samples over time.

Endogenous metabolism is related to the function of cells under starvation conditions and can be quantified with more ease than maintenance energy. While endogenous metabolism is not identical to maintenance energy, it can be used as a close estimate. Because both a single culture of protozoa and a mixed culture must be separated from bacteria for short-term measurements, the initial aim of my project was to derive and verify the efficacy of a method for separating and retaining viability of protozoa from bacteria and feed particles present in single-species cultures.

## REVIEW OF LITERATURE

### ***Studies of Endogenous Metabolism of Rumen Protozoa***

As mentioned previously, understanding maintenance energy is important for prediction of growth efficiency and thus better estimation of protein requirements of and utilization by ruminant livestock. Although rumen bacterial maintenance energy requirements have been well-studied, the same is not true for rumen protozoa. Prins and Van Hoven (1977) studied

endogenous metabolism in *Isotricha prostoma* by measuring endogenous breakdown of amylopectin that had been previously synthesized during incubation periods of varying length. Endogenous breakdown varied between 51 and 66 pmol amylopectin cell<sup>-1</sup> h<sup>-1</sup>. These results varied based on the energy source present for fermentation, and the authors assumed that the rate of endogenous amylopectin fermentation depended on the initial amount stored within the cell after starvation. Van Hoven and Prins (1977) conducted similar studies with *Dasytricha ruminantium*, another species of rumen ciliate protozoa, and measured endogenous metabolism in the same manner. Breakdown of amylopectin averaged 10.9 and 12.5 pmol cell<sup>-1</sup> h<sup>-1</sup> for two separate experiments, and these rates were much lower than for *I. prostoma*, which likely stems from the larger size of the latter.

Hillman et al (1995) also studied endogenous metabolism of rumen protozoa by evaluating the effects of feed type and time after feeding. They separated four protozoal fractions (mixed populations, large entodiniomorphs, *Isotricha spp.*, and *Dasytricha spp.*) from rumen fluid of sheep and evaluated the rate of uptake of glucose and the production of acetate, butyrate, L-lactate, and total gases by these groups of protozoa. Their method of separating protozoal fractions via use of defined-aperture textiles appeared effective, but they did not report yields of protozoa and did not study the small entodiniomorphid fraction, which generally comprises the majority of protozoa in the rumen. Most importantly, they did not examine production of D-lactate or propionate by any of the protozoal groups, and as such their estimation of endogenous activity is likely artificially low.

### ***Use of Endogenous Metabolism in Modeling of Rumen Dynamics***

Dijkstra (1994) published a mathematical model that was modified from his original model (Dijkstra et al., 1992) that simulated the dynamics of nutrient input and microbial pool sizes in the rumen. The 1994 model placed more focus on rumen protozoa, which had been recognized to play more of a role in degradation of feed than originally thought. The model contained 19 state variables representing various nutrient and microbial pools within the rumen and considered specific characteristics and requirements of protozoa, including preference for starch and sugar over fiber, predation and engulfment of bacteria and other protozoa, selective retention, and lysis based on nutrient availability.

Dijkstra's (1994) model takes protozoal maintenance requirements into account; however, the value of hexose required per day was derived by using the range of values for endogenous metabolism of amylopectin determined by Prins and Van Hoven (1977) and Van Hoven and Prins (1977) and choosing a number that fell within that range and was additionally slightly higher than the maintenance requirement for mixed rumen bacteria determined by Isaacson et al. (1975). This is problematic because the methods used by Prins and Van Hoven and Van Hoven and Prins to filter and separate protozoa from bacteria are less than ideal, as will be described in a later section. Additionally, those endogenous metabolism requirements were determined for isotrichid protozoa, which comprise only a small portion of the total protozoal pool in the rumen, and as such cannot reliably predict maintenance requirements for mixed rumen protozoa.

Finally, Dijkstra's (1994) model is highly sensitive to changes in the maintenance requirement for protozoa; for example, increasing the requirement by 50% completely extirpated simulated protozoa, and decreasing it by 25% had the same effect on cellulolytic bacteria. A subsequent model adapted certain aspects of this earlier model but still relied on empirical fitting (Hook et al., 2012) rather than mechanistic measurements that have not yet been derived and are

the ultimate objectives of this research. Clearly, the protozoal maintenance requirement is highly important for correctly predicting protein requirements for ruminant livestock and must be determined more accurately to improve modeling of rumen microbe dynamics and nutrient use.

### ***Methods for Isolating Rumen Protozoa***

Rumen protozoal maintenance energy has been much less studied than that of bacteria, largely due to the difficulty in preparing pure samples for experimentation. Protozoa predate bacteria and, as such, require them to be present in culture. Besides the limitation that a small and unknown proportion of strains of rumen protozoa can be maintained in culture, the main difficulty lies in separating protozoa from bacteria in order to measure maintenance requirements without contamination. Prins and Van Hoven (1977) prepared suspensions of *Isotricha prostoma* directly from rumen fluid by collecting sedimented protozoa from a cylindrical separatory funnel and washing with anaerobic buffer. Cell suspensions were incubated with penicillin-G overnight as well as being treated with ampicillin before endogenous metabolism experiments. The authors describe counting procedures for protozoa and state that the antibiotics used are satisfactory for preventing bacterial growth; however, they did not provide numbers for bacterial contamination and assumed that all bacteria would be killed by the time their measurements were made.

Van Hoven and Prins (1977) used similar techniques for isolating *Dasytricha ruminantium* from rumen fluid and mentioned the use of a paint brush to remove the protozoa from the glass sides of the separatory funnel. This sedimentation technique is problematic because it would likely decrease recovery of protozoa and would especially not be applicable to other species of protozoa such as the predominant *Entodinium spp.*, which tend to remain suspended in fluid much more readily. Protozoa were also starved overnight, which is an

unphysiological condition. Finally, the authors did not report data for recovery of protozoa, which weakens the validity of their separation technique for exclusive quantification purposes.

Improved methods for separation of rumen protozoa from feed particles and bacteria have since been described. Williams and Yarlett (1982) separated *D. ruminantium* and mixed isotrichids via the use of defined aperture nylon textiles and a modified Hartley filter, which increased overall viability and metabolic activity of the protozoa, as determined by measuring glucose uptake and subsequent metabolite formation. This method also allowed for faster separation and reduced oxygen exposure for microbes, although it was not completely anaerobic. Sylvester et al. (2005) measured bacterial contamination of rumen fluid samples filtered using nylon textiles and found it to be much lower than when using the sedimentation technique.

Coleman (1992) used a similar technique to isolate *Entodinium* species (from single-species cultures) from starch granules and cellulose particles after experimentation, although original protozoal suspensions were prepared by sedimentation, which is not as effective as filtration. Additionally, Coleman did not report bacterial contamination, protozoal recovery, or important details (such as buffer volumes) necessary to repeat the procedure. To study maintenance requirements of protozoa, which play an important role in rumen microbial dynamics, an efficient and reliable method of separation from feed particles and bacteria is needed.

## MATERIALS AND METHODS

### ***Protozoal Culture and Rumen Fluid Collection***

The *E. caudatum* (Stein, 1859) culture used in this study was previously established by Dehority (2008). It originated from a single cell picked from rumen fluid of a gerunuk antelope



housed at the St. Louis Zoo. Culture was maintained in 1-L stoppered Roux flasks in volumes of 320 mL per flask (with one flask for each of three growth rates: 0.014/h, 0.021/h, and 0.042/h) and cultured using materials and methods as described by Dehority (1998). Controlled growth rates were achieved by replacing half the culture with an equal volume of fresh Media-SP every 72, 48, and 24 h, respectively. Cultures were fed 3.8 mL of a 1.5% wheat, 1% orchardgrass (ground to fit through a 40- $\mu$ m mesh screen) suspension in distilled water every 24 h.

Rumen fluid for experiments with mixed rumen protozoa was collected from 1 of 2 cannulated Jersey cows fed a lactation diet (50% corn silage, 4.5% alfalfa hay, 21% corn wet milling product [Cargill Corn Milling, Dayton, OH], 9.05% ground corn, 4.64% soybean meal, 1.30% Amino Plus [Ag Processing Inc. Hiawatha, KS], 1.30% soyhulls, 0.38% fat, 2.01% vitamin and minerals) at *ad libitum* in 2 equal meals. At 2.5 h after feeding, rumen contents were strained through 4 layers of cheesecloth. The strained fluid (1000 mL) was diluted 1:1 with N-free buffer [Simplex type, pH = 6.8 (3)] and added to a separatory funnel. Feed particles were removed by aspiration after flocculating for 45 min at 39°C.

### ***Filtration of Protozoal Culture and Rumen Fluid***

*E. caudatum* cells in 50 mL aliquots of culture were separated from feed particles and bacteria under a constant stream of CO<sub>2</sub> via use of a filtration apparatus (Figure 1) and stepwise filtration through 2 nylon screens of decreasing aperture. Protozoa were separated from a) feed particles via filtration through a 35- $\mu$ m screen with buffer (37.5 mL) and b) bacteria via filtration of filtrate from previous step through a 10- $\mu$ m screen with buffer (75 mL). Previous work in the lab indicated that the mean length of *E. caudatum* = 41.5 (6.1 SD)  $\mu$ m and mean width = 26.7 (1.8 SD)  $\mu$ m. Screen sizes were chosen based on the requirement that the filter for step a) must

be sufficiently larger than the width of the protozoa to ensure complete passage through the screen and that the filter pore size for step b) must be significantly smaller than protozoal width to ensure completeness of recovery. The protozoal retentate (4 mL) from the second filtration was centrifuged at 1000 x *g* for 5 min and resuspended to a final volume of 2 mL. One milliliter was saved for determining heat production, 300 µL stored at -20°C for protein analysis, and 250 µL diluted to 500 µL with 50% formalin and saved for counting to determine protozoal cell recovery and bacterial contamination. *E. caudatum* cultures were treated one of 3 ways during filtration in order to determine the effect, if any, of feeding time on heat production. Cultures were fed either 24 h (starvation) or 0.75 h pre-filtration or immediately post-filtration; these treatments were run in duplicate.

Mixed rumen protozoa were separated from rumen fluid using the same basic filtration procedure as described above, with a few modifications. First, feed particles were removed by aspiration (see above) rather than filtration. Retentate was then filtered through a 20-µm nylon screen for removal of contaminating bacteria as opposed to a 10-µm screen. Retentate was not centrifuged but rather collected directly from the 20-µm screen into a final volume of 20 mL and saved in aliquots as described above. Due to the inability to culture mixed rumen protozoa, the only treatment utilized for these experiments was the difference in cows from which fluid was collected. Three separate experiments with mixed rumen protozoa were performed.

### ***Determining Heat Production of Protozoa***

Rate of heat production was measured using isothermal microcalorimetry (Thermal Hazard Technology µRC; Piscataway, NJ) as described by Hackmann et al. (2013). Briefly, cell suspension prepared as described above (1 mL) was added to a 2-mL autosampler vial with a

rubber septum and placed in the measurement cell. Water (1 mL) was placed in the reference cell, and the calorimeter was set to 39.00°C. Heat production was recorded at intervals of 1 s. Rate of heat production was corrected for a baseline obtained by injecting 1 mL 1% formalin into the sample cell after sample equilibration and allowing heat production to stabilize again. Owing to extremely long equilibration times and difficulty establishing baselines using this method, likely due to temperature fluctuations in the laboratory, the method of establishing baselines was changed after several experiments to use of water as a blank in the sample and reference cells before and after each experiment.

### ***Determining Protein Content, Cell Numbers, and Protozoal Viability***

Protein was determined using the Pierce BCA Protein Assay Kit (product #23227; Thermo Scientific, Rockford, IL) after hydrolyzing the pellet in NaOH (0.2 N final concentration, 100°C, 15 min). Protozoal cell counts were performed on formalinized samples using a Sedgewick-Rafter chamber (Dehority, 1993). Direct bacterial cell counts were performed as described by Holdeman and Moore (1972). Both protein and cell counts were measured before and after filtration to determine recovery of protozoal cells and contamination by bacteria; these assays were also used to scale heat production per gram of microbial protein. Protozoal viability was determined by examining samples microscopically and observing movement, cell membrane integrity, and overall appearance of cells.

## **RESULTS**

### ***Filtration Procedure***

Filtration of *E. caudatum* using the filtration apparatus in Figure 2 resulted in 51 (18.7 SD) % protozoal cell recovery ( $n = 21$ ) as determined by counting of samples taken before and after filtration. Post-filtration samples were determined to be 70 (9.6 SD)% viable ( $n = 21$ ) and contained only 1.89 (0.18 SD)% bacterial contamination ( $n = 3$ ). Previous work in the lab indicated that protein mass per bacterial cell was  $1.34 \cdot 10^{-10}$  ( $2.18 \cdot 10^{-11}$  SD) mg/cell ( $n = 8$ ). This was determined by centrifuging *E. caudatum* culture at  $1000 \times g$  for 5 min to pellet protozoa and then centrifuging supernatant at  $10,000 \times g$  for 10 min, washing with 0.9% saline, and centrifuging at  $10,000 \times g$  for 10 min to pellet bacteria. Bacterial samples were analyzed with BCA protein assay to determine protein content per milliliter and divided by number of cells per milliliter. Bacterial contamination on a protein basis was expressed as a percentage of total microbial protein.

Figure 3 compares protozoal culture before and after filtration, with the resulting sample being almost completely free of feed particles and bacteria. Total filtration time averaged approximately 20 min (data not shown) and seemed to be sufficiently rapid to prevent excessive cell death.

### ***Heat Production by E. caudatum***

A representative graph of protozoal heat production over time for growth rate of 0.014/h is shown in Figure 4. Note that this fractional rate is a proportion of turnover of the protozoal cell turnover (includes cell division corrected for any potential cell lysis). Heat production for protozoa fed 0.75 h pre-filtration and corrected for a baseline established using water blanks averaged 7.1 (2.1 SD) mW/g microbial protein ( $n = 2$ ); ratio of protozoal heat production signal to baseline noise =  $< 3$ . Because heat production was much lower than the expected value of 30-

40 mW/ g microbial protein and would be expected to further decrease at higher growth rates, heat production was not evaluated for *E. caudatum* cultures at growth rates 0.021/h and 0.042/h. Because the use of 1% formalin to establish a baseline proved unsuccessful, heat production data from the starvation and feeding post-filtration treatments is not publishable. However, there was no apparent difference in heat production across the three treatments.

### ***Heat Production by Mixed Rumen Protozoa***

A representative graph of heat production by mixed rumen protozoa is shown in Figure 5. Heat production averaged 31.4 (8.4 SD) mW/ g microbial protein ( $n = 3$ ), and the ratio of the protozoal heat production signal to baseline noise was  $> 10$  for each experiment. This value of endogenous heat metabolism is lower than, but still closer to, the expected value when compared with *E. caudatum*.

## **DISCUSSION**

The purpose of this study was to improve a method for filtering protozoa from a single-species culture and from rumen fluid and to determine maintenance energy and endogenous metabolism requirements for *Entodinium caudatum* and mixed rumen protozoa, respectively. The filtering method described above resulted in viable samples of protozoa with very little contamination by bacteria. Recovery of protozoal cell numbers could be improved, likely by washing more thoroughly with buffer or more extensive pipetting from the filter, but there must be a balance between completeness of recovery and speed of filtering; protozoa are more likely to die the longer they sit on the filter, out of media, and at a much lower temperature than is required. This filtering method is also desirable because it can be used for separating virtually

any size of protozoa; it has been used for filtering single-species cultures of the much larger *Epidinium caudatum* with similar results (data not shown).

Heat production of *Entodinium caudatum* was marginally above the baseline and less than one-fourth the predicted value of 30-40 mW/ g microbial protein across all experiments at growth rate 0.014/h. As mentioned, heat production at higher growth rates was not evaluated because it would be expected to be even lower. As cells increase their rate of growth, they devote more energy towards growth and less towards maintenance of their own cells; as such, protozoa growing at a rate of 0.021/h or 0.042/h would likely produce less heat than those growing at 0.014/h. Because the signal:noise ratio (SNR) was  $< 3$  for these experiments, the heat production of *E. caudatum* measured to be 7.1 mW/g microbial protein is not different from zero because this value statistically could be a result of noise from the instrument. Although it is tempting to speculate that *E. caudatum* simply has a much lower maintenance requirement than originally estimated, further work must be performed and other techniques utilized to determine the correct value. Filtering larger volumes of culture might improve the accuracy of heat production measurement, but these volumes might prove to be too large to realistically maintain in a laboratory setting.

Heat production by mixed rumen protozoa was close to 30 mW/ g microbial protein for all experiments, which is much closer to expected values. Because these samples are taken directly from the rumen, they contain more biomass and thus produce more heat. These experiments did not meet our objective for deriving the true maintenance energy because growth rates cannot be controlled. However, endogenous metabolism is a close estimate of maintenance energy because it is a reflection of heat production by unfed protozoa over time. Without feed,

protozoa are expected to divert less energy to growth and more towards cellular maintenance functions.

## CONCLUSIONS

The described method for filtering rumen protozoa was efficient in separating protozoa of varying sizes from feed particles and bacteria present in either culture or rumen fluid while maintaining protozoal viability. Heat production of *E. caudatum* was not significant from zero, so a specific maintenance requirement cannot yet be given for this species. However, endogenous metabolism of mixed rumen protozoa was close to (though slightly lower than) the predicted value and provides a starting point for future studies of protozoal maintenance requirements.

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Figure 1. ATP energy directed towards maintenance and growth efficiency of bacteria.

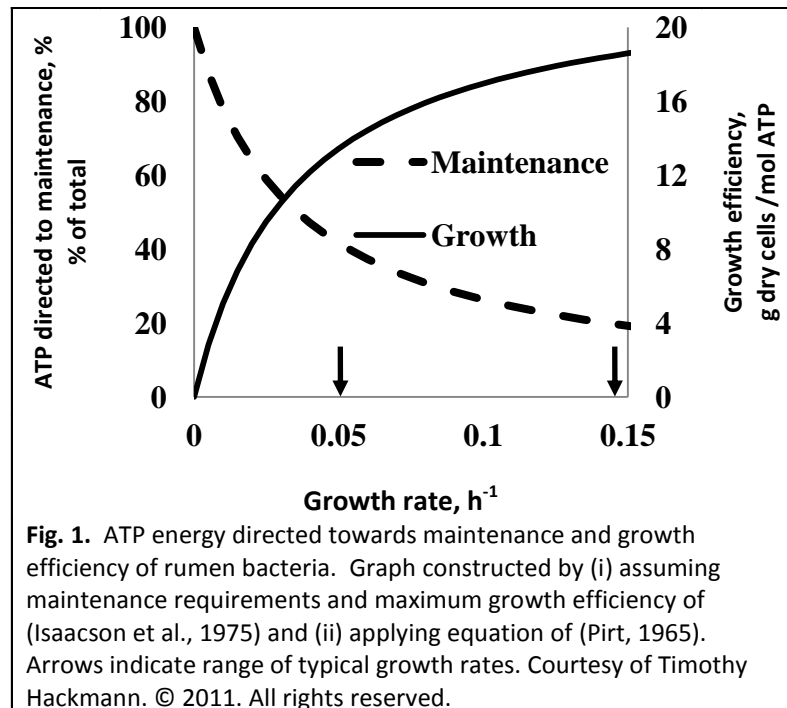


Figure 2. Protozoal filtration apparatus.

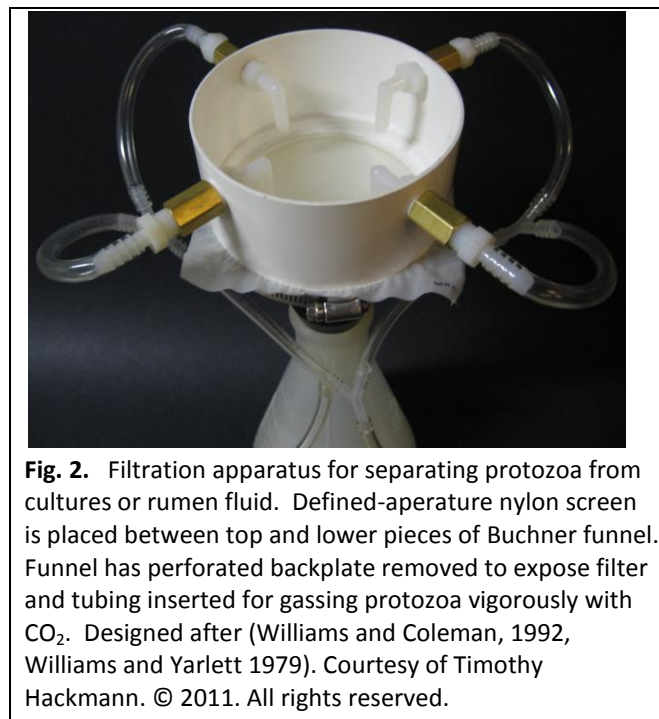


Figure 3. *Entodinium caudatum* culture pre- and post-filtration.

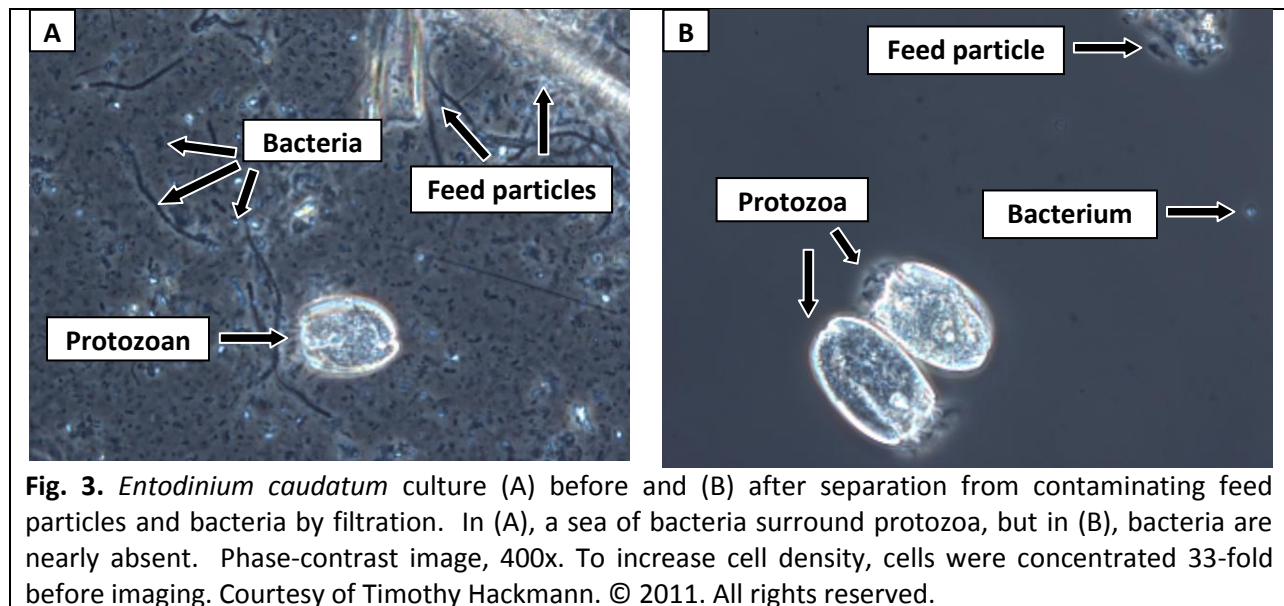


Figure 4. Heat production of *Entodinium caudatum*, growth rate = 0.014/h.

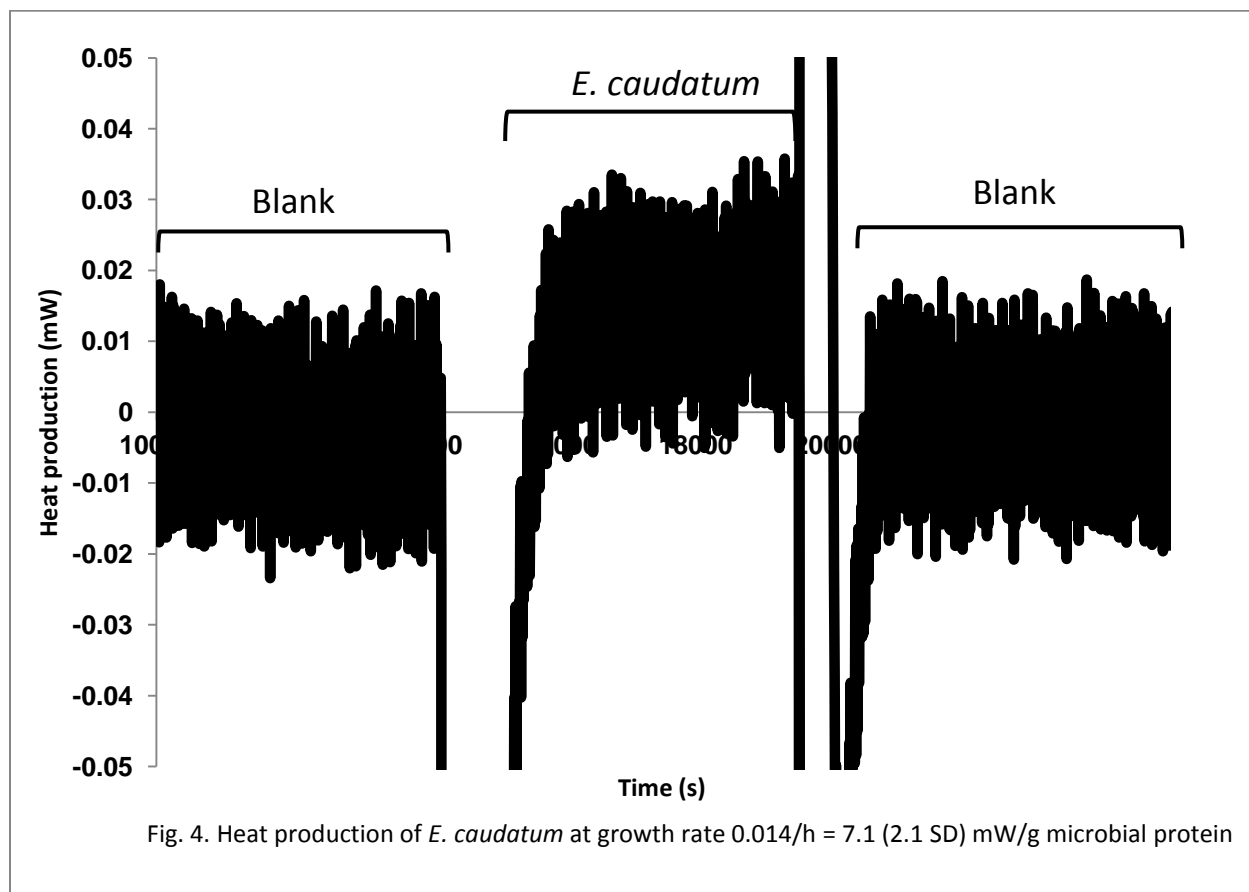


Figure 5. Heat production of mixed rumen protozoa.

